Ellagic acid inhibits lipopolysaccharide-induced expression of enzymes involved in the synthesis of prostaglandin E₂ in human monocytes

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Ellagic acid, a natural polyphenol found in certain fruits, nuts and vegetables, has in recent years been the subject of intense research within the fields of cancer and inflammation. Pain, fever and swelling, all typical symptoms of inflammation, are ascribed to elevated levels of PGE_2. In the present study, we have investigated the effects of ellagic acid on PGE_2 release and on prostaglandin-synthesising enzymes in human monocytes. Ellagic acid was found to inhibit Ca ionophore A23187-, phorbol myristate acetate- and opsonised zymosan-induced release of PGE_2 from monocytes pre-treated with the inflammatory agent lipopolysaccharide. Ellagic acid suppressed the lipopolysaccharide-induced increase in protein expression of cyclo-oxygenase-2 (COX-2), microsomal PGE synthase-1 (mPGEs-1) and cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$), while it had no effect on the constitutively expressed COX-1 protein. Ellagic acid had no apparent inhibitory effect on these enzymes when the activities were determined in cell-free assays. We conclude that the inhibitory effect of ellagic acid on PGE_2 release from monocytes is due to a suppressed expression of COX-2, mPGEs-1 and cPLA$_2\alpha$, rather than a direct effect on the activities of these enzymes.

Ellagic acid: Prostaglandin E₂: Human monocytes

PGE_2, which belongs to the family of eicosanoids, is a potent lipid mediator that is produced in most cell types. During inflammation, increased levels of PGE_2 are seen in response to various substances such as cytokines, growth factors and bacterial agents[1]. The production of PGE_2 is dependent upon the activity of several enzymes involved in the arachidonic acid (AA) cascade. AA is liberated from cellular membrane phospholipids through the action of different types of phospholipase A_2 (PLA$_2$). This family of enzymes can be divided into three main groups: secretory PLA$_2$, Ca-independent PLA$_2$ and cytosolic PLA$_2$[2,3]. Among these, the ubiquitously expressed cytosolic PLA$_2\alpha$ (cPLA$_2\alpha$) has gained interest in eicosanoid production due to its preferential hydrolysis of AA-containing phospholipids and its activation in response to various inflammatory stimuli[4,5]. Following mobilisation, AA is subsequently converted by either the constitutively expressed cyclo-oxygenase-1 (COX-1) or the inducible COX-2 to the intermediate PGH_2, which is then further metabolised to various prostaglandins. Among these, PGE_2 is the main prostaglandin released from lipopolysaccharide (LPS)-treated monocytes[6]. PGH_2 is converted to PGE_2 by one of three different isoforms of PGE synthase (PGEs): cytosolic PGEs (cPGEs) and microsomal PGEs 1 and 2 (mPGEs-1, mPGEs-2)[7]. cPGEs and mPGEs-2 are in most cell types regarded as constitutively expressed proteins while mPGEs-1, along with COX-2, are known to be inducible[8]. COX-2 and mPGEs-1 are also considered to be the main actors when PGE_2 is produced during inflammation[9].

Ellagic acid is a polyphenolic compound of which the highest levels are found in fruits such as pomegranate, blackberries, raspberries and strawberries. Juices from these fruits have been proposed to have health-beneficial properties. Ellagic acid has been shown to inhibit proliferation and to initiate apoptosis in certain forms of cancer cell lines[10–14] as well as having anti-inflammatory effects[15,16]. The molecular mechanisms by which ellagic acid exerts its effects are largely unknown, although ellagic acid has been shown to affect protein kinases, such as mitogen-activated protein kinases[17,18] and also to modulate the activity of transcription factors, such as NFκB[16,19,20]. As a consequence of the reported positive effects from a health point of view, a more intense research concerning the exact mechanisms by which ellagic acid exerts its effects is in demand.

In the present study we have investigated the effects of ellagic acid on PGE_2 release and on expression of the enzymes involved in the synthesis of PGE_2 in human monocytes.

Materials and methods

Materials

Ficoll-Paque™ PLUS and 1-stearoyl-2[^{14}C]arachidonoyl-phosphatidylcholine were purchased from GE Healthcare (Little Chalfont, Bucks, UK). Dulbecco's

Modified Eagle Medium: Nutrient Mixture F-12 (DMEM:F12), penicillin and streptomycin and NuPAGE gels were obtained from Invitrogen (Carlsbad, CA, USA). Cellstar® twelve-well culture plates were purchased from Greiner Bio-One (Frickenhausen, Germany) and BD Falcon™ 100 mm cell-culture plates from Becton Dickinson (Franklin Lakes, NJ, USA). Ellagic acid, LPS (*Escherichia coli* serotype 055:B5), A23187, Zymosan A, phorbol myristate acetate (PMA) and propidium iodide were all purchased from Sigma-Aldrich (St Louis, MO, USA). Pyrrophenone was generously provided by Dr Kauro Seno (Shianogi & Co. Ltd, Osaka, Japan). Indomethacin, NS398, PGH₂ and AA, as well as a PGE₂ enzyme immunoassay kit, were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Primary antibodies (goat monoclonal anti-COX-2, goat monoclonal anti-COX-1, goat monoclonal anti-cPLA₂α and rabbit monoclonal anti-mPGES-1), as well as peroxidase-conjugated donkey anti-goat and donkey anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Supersignal West Pico Chemiluminescent system was obtained from Thermo Fisher Scientific (Rochester, NY, USA).

Isolation of monocytes

Fresh human monocytes were isolated from buffy coats of healthy blood donors. The buffy coat was diluted with an equal volume of PBS containing 3 mM-EDTA, and was then carefully loaded on Ficoll-Paque™ PLUS and centrifuged at 800 *g* for 30 min at 20°C. The separated mononuclear fraction was collected and diluted with PBS containing 3 mM-EDTA followed by centrifugation at 500 *g* for 15 min. The pelleted cells were re-suspended in PBS containing 3 mM-EDTA and washed five times by centrifugation at 180 *g* for 10 min. After washing, the cells were re-suspended in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM:F12; Invitrogen) culture medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 5 % (v/v) human serum. The cells were seeded onto polystyrene cell-culture plates and allowed to adhere for 2 h. Non-adherent cells were washed away with PBS containing 1 mM-CaCl₂ and 1 mM-MgCl₂, and fresh serum-free medium was added. The cells were maintained at 37°C in a humidified atmosphere of 5 % CO₂ for a total time of 2 d.

Pre-treatment of monocytes

After 1 d in culture, the monocytes were washed three times with PBS containing 1 mM-CaCl₂ and 1 mM-MgCl₂ and fresh medium was added. Cells were either left untreated (control) or pre-treated with LPS (1 μg/ml) for 20 h with or without combined pre-treatment with various concentrations of ellagic acid. Freshly made ellagic acid, dissolved in dimethyl sulfoxide to a concentration of 10 mM, was added to the cells 1 h before LPS. An equal volume of dimethyl sulfoxide, with no ellagic acid, added to control cultures had no effect on any of the experimental read-outs measured in the present study. Cell viability was determined by propidium iodide staining (incubation of cells with 50 μg/ml for 15 min) and counting of stained cells using a fluorescence microscope with 545 nm excitation and 605 nm emission filters.

Immunoblotting

The cells (approximately 8 × 10⁶ cells) were scraped in 150 μl Laemmli sample buffer[21] containing leupeptin (1 μg/ml), aprotinin (1 μg/ml), 1 mM-phenylmethylsulfonyl fluoride and 5 % (v/v) β-mercaptoethanol and boiled for 5 min. A quantity of 25 μl of lysates was loaded to NuPAGE 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) glycine gradient gel (4–12 %) and separated proteins were electrotransferred onto polyvinylidene difluoride membranes. The membranes were blocked with 2·5 % (v/w) gelatine in Tris-buffered saline (TBS) containing 0·1 % (v/v) Tween-20 for 1 h at room temperature, washed two times in TBS–Tween-20 and incubated overnight at 8°C with primary antibodies: goat monoclonal anti-cPLA₂α (1:500), goat monoclonal anti-COX-2 (1:300), goat monoclonal anti-COX-1 (1:300) or rabbit monoclonal anti-mPGEs-1 (1:250). The membranes were washed five times for 10 min in TBS–Tween-20 and thereafter incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibodies: donkey anti-goat IgG (1:4000) for cPLA₂α, COX-1 and COX-2 and donkey anti-rabbit IgG (1:1500) for mPGEs-1. The membranes were washed five times with TBS–Tween-20 and proteins were detected with the Supersignal West Pico Chemiluminescent system and captured on X-ray film. Films were scanned and band intensity was analysed using ImageQuant TL (GE Healthcare). The relative band intensities of cPLA₂α, COX-2 and mPGEs-1 were normalised to the corresponding band of the constitutively expressed COX-1.

Analysis of PGE₂ release from intact monocytes

For analysis of PGE₂ release, monocytes were cultured on twelve-well plates (approximately 0·5 × 10⁶ cells per well). After pre-treatment for 20 h with LPS, with or without ellagic acid, the cells were washed three times and fresh medium was added to the wells. The cells were then allowed to equilibrate for 30 min before medium once more was exchanged with 1 ml pre-warmed (37°C) medium. Cells were stimulated with 0·5 μM-A23187, 150 nM-PMA or opsonised zymosan (0·5 mg/ml) for 30, 45 and 60 min respectively. Culture medium from each well was transferred into collecting tubes and centrifuged at 3000 rpm for 2 min. The amount of released PGE₂ was determined with a monoclonal PGE₂ enzyme immunoassay kit according to the manufacturer's instructions. Results, pg released PGE₂ per ml medium, are expressed as percentage of maximum release for each stimuli and standard errors.

Isolation of subcellular fractions

Monocytes for preparation of subcellular fractions were cultured on 100 mm cell-culture plates (approximately 8 × 10⁶ cells per plate) and treated as described above. After washing the plates three times with PBS, cells from three culture plates were scraped off in a total volume of 1 ml ice-cold Buffer X (80 mM-KCl, 1 mM-EDTA, 10 mM-HEPES, pH 7·4) containing leupeptin (1 μg/ml), aprotinin (1 μg/ml) and 1 mM-phenyl-methylsulfonyl fluoride. The cell suspensions were sonicated with a probe sonicator for 2 × 5 s at 4°C and centrifuged at 1700 *g* for 10 min at 4°C. Supernatant fractions were

transferred into centrifugation tubes and centrifuged at $60\,000\,g$ for 90 min at 4°C. The supernatant fractions (cytosolic fractions) were collected and glycerol was added to a final concentration of 10 % (v/v) and stored at 4°C until assayed for $cPLA_2\alpha$ activity. The pellets (microsomal fractions) were re-suspended in 500 μl Buffer X containing leupeptin (1 μg/ml), aprotinin (1 μg/ml) and 1 mM-phenyl-methylsulfonyl fluoride and sonicated $2 \times 5\,s$ at 4°C. Microsomal fractions were stored at −20°C until assayed for mPGEs activity. The total amount of protein in each subcellular fraction was determined by the method of Bradford[22] using bovine serum albumin as standard.

Assay of combined cyclo-oxygenase and PGE synthase activity

A quantity of 15 μl of supernatant fractions from $1700\,g$ centrifugation was mixed with Buffer X containing 2 mM-glutathione in a total volume of 100 μl. In experiments where the direct effect of ellagic acid on enzyme activity was determined, ellagic acid was added to the reaction mixtures and pre-incubated for 15 min. Reactions were started by the addition of 0·5 μM-AA, dissolved in ethanol. After 30 min incubation at 37°C, the reactions were terminated by the addition of 400 μl of ice-cold methanol and centrifuged at $10\,000\,g$ for 10 min at 4°C. Supernatant fractions were transferred into new collection tubes and dried under N_2 gas, re-dissolved in culture medium and assayed for PGE_2 with an enzyme immunoassay kit. The activity detected with this assay was inhibited by 100 (SEM 0) % and 86 (SEM 6) % (*n* 3) with 10 μM-indomethacin and 20 μM-NS398, respectively. Results are expressed as pg PGE_2 per μg protein and standard errors.

Assay of microsomal PGE synthase activity

A quantity of 70 μl of microsomal fractions was mixed with 30 μl Buffer X containing 2 mM-glutathione and 10 μM-indomethacin. The mixtures were incubated for 15 min, with or without the addition of ellagic acid, before the reaction was initiated by the addition of 0·5 μg of PGH_2 dissolved in ethanol. The enzyme reaction was allowed to proceed for 60 s at room temperature and was terminated by the addition of 25 μl 100 mM-$FeSO_4$. Appropriate dilutions in culture medium were made for each reaction and assayed for produced PGE_2 using an enzyme immunoassay kit. One reaction was assayed without enzyme source and considered as a control of non-enzymic conversion of PGH_2 and this value was subtracted. Results are expressed as pg PGE_2 per μg protein and standard errors.

Assay of cytosolic phospholipase $A_2\alpha$ activity

A quantity of 50 μl of cytosolic fractions was assayed with sonicated vesicles of 1-stearoyl-2[^{14}C]arachidonoyl-phospha-tidylcholine (100 pmol) as substrate and 1 mM-free Ca^{2+} in a total volume of 525 μl of Buffer X. In experiments where the direct effect of ellagic acid on $cPLA_2\alpha$ activity was investigated, ellagic acid was added to the reaction mixtures and pre-incubated for 15 min. Incubations were terminated after 30 min at 37°C by the addition of 2 ml chloroform–methanol–10 M-HCl (2:1:0·01, by vol.) and by the addition

of carrier lipids (unlabelled AA and phosphatidylcholine). After centrifugation at 3000 rpm for 10 min, the lipid phase was subjected to silicic acid (200 mg) column chromatography. Fatty acids were eluted with chloroform ($2 \times 0·5$ ml) and phospholipids with methanol (3×1 ml) and radioactivity determined by scintillation counting. The activity detected with this assay was inhibited by 92 (SEM 6) % (*n* 5) with 1 μM-pyrrophenone, a selective inhibitor of $cPLA_2\alpha$[23]. Results are expressed as pmol hydrolysed substrate per μg protein and standard errors.

Statistical analysis

Statistical analysis was performed using Student's paired two-sided *t* test. $P < 0.05$ was considered significant.

Results

Effect of ellagic acid on PGE_2 release in human monocytes

Human monocytes, pre-treated with LPS (1 μg/ml) for 20 h, responded to a subsequent stimulation with either A23187, PMA or opsonised zymosan with a considerable release of PGE_2 that amounted to 2·5–3·5 ng/ml. This PGE_2 release is about 100-fold higher than that seen without LPS pre-treatment in response to the same stimuli (results not shown). PGE_2 release was also seen in response to LPS alone; however, this release was low (0·1 (SEM 0·04) ng/ml (*n* 5)) and subsequent experiments were therefore performed using the experimental set-up with LPS pre-treatment followed by stimulation with A23187, PMA or opsonised zymosan. Ellagic acid, added 1 h before pre-treatment with LPS, inhibited the PGE_2 release induced by A23187, opsonised zymosan or PMA with a half maximal inhibition at 10–15 μM-ellagic acid (Fig. 1). Ellagic acid at 30 μM inhibited the release seen with A23187, opsonised zymosan and PMA with 86·2 (SEM 5·3) % (*n* 6), 92·7 (SEM 1·8) % (*n* 6) and 83·9 (SEM 3·6) % (*n* 4), respectively. In contrast to the inhibition seen when ellagic acid was added before LPS pre-treatment, no inhibition of PGE_2 release was seen when ellagic acid was added 15 min before stimulation with A23187, opsonised zymosan or PMA (results not shown).

Ellagic acid, at concentrations up to 20 μM, had no effect on cell viability as determined by propidium iodide staining, while 30 μM revealed a 4·8 (SEM 3·1) % (*n* 4) and 50 μM-ellagic acid more than 10 % loss in cell viability.

Effect of ellagic acid on the lipopolysaccharide-induced protein expression of prostaglandin-synthesising enzymes

Next, we investigated if the effect of ellagic acid on PGE_2 release was due to an impact on protein expression of the involved prostaglandin-synthesising enzymes: COX-1, COX-2, mPGEs-1 and $cPLA_2\alpha$. Whole cell lysates, from resting monocytes as well as monocytes treated with LPS, with or without various concentrations of ellagic acid, were subjected to electrophoresis and immunoblotting. COX-1 protein was present in control cells and was not affected by LPS treatment or any used dose of ellagic acid (Fig. 2(a)). COX-2 protein was not detected in control cells, but, was markedly induced by LPS treatment (Fig. 2(b)).
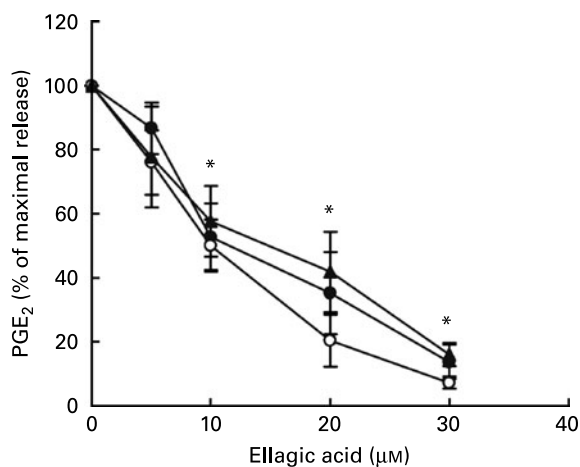
Fig. 1. Dose-dependent inhibitory effect of ellagic acid on PGE$_2$ release from monocytes. Cells were pre-treated with lipopolysaccharide (LPS; 1 μg/ml) for 20 h with or without indicated concentrations of ellagic acid (ellagic acid was added 1 h before LPS). Cells were washed and given fresh medium followed by stimulation with either 0·5 μM-A23187 (●), opsonised zymosan (0·5 mg/ml; ○) or 150 nM-phorbol myristate acetate (▲) for 30, 60 and 45 min, respectively. The culture medium was analysed for PGE$_2$ release by enzyme immunoassay and results are expressed as percentage of maximal release for each stimuli. Values are means from four to six individual experiments, with standard errors represented by vertical bars. * Significant inhibition compared with maximal release for each stimuli ($P < 0.05$).

This augmented expression of COX-2 protein was reduced by treatment with ellagic acid: a 50 % reduction in the LPS-induced expression of COX-2 protein was seen at 30 μM-ellagic acid. Regarding mPGEs-1 protein, this could be detected in control cells; however, the expression was increased about five-fold in response to LPS treatment (Fig. 2(c)). Ellagic acid suppressed the LPS-induced increase in expression of mPGEs-1 and an 80 % inhibition was seen at 30 μM-ellagic acid. cPLA$_2$α protein was detected in control cells and the expression was further increased by treatment with LPS (Fig. 2(d)). Ellagic acid inhibited the LPS-induced increase in cPLA$_2$α protein down to the level seen in control cells and this inhibition was seen already at 10 μM-ellagic acid.

Activity of prostaglandin-synthesising enzymes in subcellular fractions from control, lipopolysaccharide- and ellagic acid plus lipopolysaccharide-treated cells

In addition to the protein expression of COX, mPGEs-1 and cPLA$_2$α, the activity of these enzymes was determined using subcellular fractions from control, LPS- and ellagic acid plus LPS-treated cells. Supernatant fractions from 1700 **g** centrifugation of cell lysates were assayed for combined activity of COX and PGEs, measured as the conversion of exogenously added AA to PGE$_2$. Lysates from LPS-treated cells resulted in a ten-fold increase in the combined activity of COX and PGEs compared with lysates from control cells and this increase in activity was inhibited by 80 % in lysates from ellagic acid plus LPS-treated cells (Fig. 3(a)). mPGEs activity was determined in microsomal fractions by analysing the conversion of exogenous PGH$_2$ to PGE$_2$. As shown in Fig. 3(b), the mPGEs activity in microsomal fractions from

LPS-treated cells was increased by a factor of 3 as compared with that seen from control cells. The mPGEs activity in microsomal fractions from cells treated with 30 μM-ellagic acid plus LPS was reduced by 85 % compared with that seen from LPS-treated cells. For measurements of cPLA$_2$α activity, cytosolic fractions were analysed using sonicated vesicles of 1-stearoyl-2[^{14}C]arachidonoyl-phosphatidylcholine as substrate. As shown in Fig. 3(c), LPS treatment of cells enhanced the cPLA$_2$α activity by a factor of 1·3 compared with the activity seen in cytosolic fractions from control cells. Treatment of cells with 30 μM-ellagic acid resulted in inhibition of the LPS-induced increase in cPLA$_2$α activity down to the activity seen in cytosolic fractions from control cells.

Effects of ellagic acid on the activity of cyclo-oxygenase, microsomal PGE synthase and cytosolic phospholipase A$_2$α in a cell-free assay

To investigate if ellagic acid has any direct effect on the activity of COX, mPGEs and cPLA$_2$α, subcellular fractions were pre-treated with ellagic acid before analysis of the activity of these enzymes. Addition of ellagic acid had no direct effect on the enzymic activity of mPGES and cPLA$_2$α (Fig. 4). Somewhat surprisingly, addition of ellagic acid (10–30 μM) resulted in a two-fold increase in the combined activity of COX and PGEs (see Discussion). Although subcellular fractions from LPS-treated cells were used in the set of experiments shown in Fig. 4, the same results were seen using subcellular fractions from control cells, that is, no effect on the activity of mPGEs and cPLA$_2$α and a two-fold increase in the combined COX and PGEs activity (results not shown).

Discussion

We have investigated the effect of ellagic acid on PGE$_2$ release in human monocytes as well as its effects on protein expression and enzymic activity of cPLA$_2$α, COX and mPGEs-1. The experimental set-up in the present study, treatment of monocytes with LPS for 20 h, resulted in expression of COX-2 protein, an increase in the expression of mPGEs-1 and, also to some extent, an increase in the expression of cPLA$_2$α. We found that the PGE$_2$ release induced by A23187, opsonised zymosan and PMA stimulation of LPS-pre-treated monocytes was inhibited by ellagic acid in a concentration-dependent manner with an 80–90 % inhibition at 30 μM-ellagic acid. When comparing this with the effects of ellagic acid on the expression of involved enzymes, we found no effect of ellagic acid on the expression of COX-1 protein, suggesting that this isoform of COX does not, to any major extent, contribute to the PGE$_2$ release seen under our conditions. In contrast, ellagic acid inhibited the LPS-induced expression of COX-2 protein, with a 50 % inhibition at 30 μM-ellagic acid. mPGEs-1 is considered to be an inducible enzyme[9,24]; however, we found a low but consistent expression in control monocytes. The existence of mPGEs-1 in control cultures has also been seen in other cell types such as fibroblasts[25] and microglia cells[26]. The protein expression of mPGEs-1 was up-regulated in response to LPS treatment and this increase was reduced by ellagic acid,

with an 80 % inhibition at 30 μM-ellagic acid. We have not investigated the effect of ellagic acid on the expression of the two other isoforms of PGEs: cPGEs and mPGEs-2. However, based on other reports, these isoforms are less likely to be involved in the PGE$_2$ release under the conditions

applied in the present study. First, the expression of cPGEs and mPGEs-2 have been shown not to be affected by LPS treatment of microglia cells[26] and liver macrophages[27] or by IL-1β treatment of gastric fibroblasts[28]. Second, macrophages from mPGEs-1 knockout mice produced substantially reduced levels of PGE$_2$ compared with macrophages from wild-type mice[29–31]. In addition to effects on COX-2 and mPGEs-1 protein, we also found ellagic acid to inhibit the LPS-induced increase in cPLA$_2$α protein. Thus, ellagic acid reduced the LPS-induced increase in protein expression of all three enzymes involved in the synthesis of PGE$_2$ and this was accompanied by a reduction in the activity of the enzymes when assaying subcellular fractions from cells treated with ellagic acid plus LPS compared with LPS alone.

Although ellagic acid reduced the LPS-induced increase in expression of all three enzymes, the present results support a role for COX-2 and mPGEs-1, but not cPLA$_2$α, to be rate limiting in the synthesis of PGE$_2$ in monocytes. This is based on the observations that COX-2 and mPGEs-1 protein expression showed a tendency to being concentration-dependently inhibited by ellagic acid in the range of 10–30 μM and this was similar to the inhibition of the PGE$_2$ release. In contrast, cPLA$_2$α protein was maximally affected already at 10 μM-ellagic acid while PGE$_2$ release continued to decline with 20 and 30 μM-ellagic acid.

In order to exclude a direct inhibitory effect of ellagic acid on the activity of COX, mPGEs and cPLA$_2$α, we also performed experiments where ellagic acid was added to subcellular fractions for measuring enzymic activity. mPGEs and cPLA$_2$α activity was not affected by the addition of ellagic acid while it increased the combined activity of COX and PGEs. We have no explanation for this increase in activity. However, due to the fact that ellagic acid had no direct effect on the mPGEs activity and that cPGEs is less likely to be involved, based on its low catalytic efficiency compared with mPGEs-1[32], we suggest that the enhancing effect of ellagic acid is due to an effect on the COX enzymes. Phenols in the low millimolar range have been shown to enhance COX activity and this effect has been attributed to stimulation of the peroxidase activity of COX as well as preventing the enzyme from self-catalysed inactivation[33]. Ellagic acid could potentially work in a similar way as phenols, in stimulating COX activity, when added directly to subcellular fractions for activity measurements.

The mechanisms by which ellagic acid inhibits the LPS-induced expression of COX-2, mPGEs-1 and cPLA$_2$α is unknown; however, it most likely involves effects on protein kinases and/or transcription factors. Ellagic acid has been
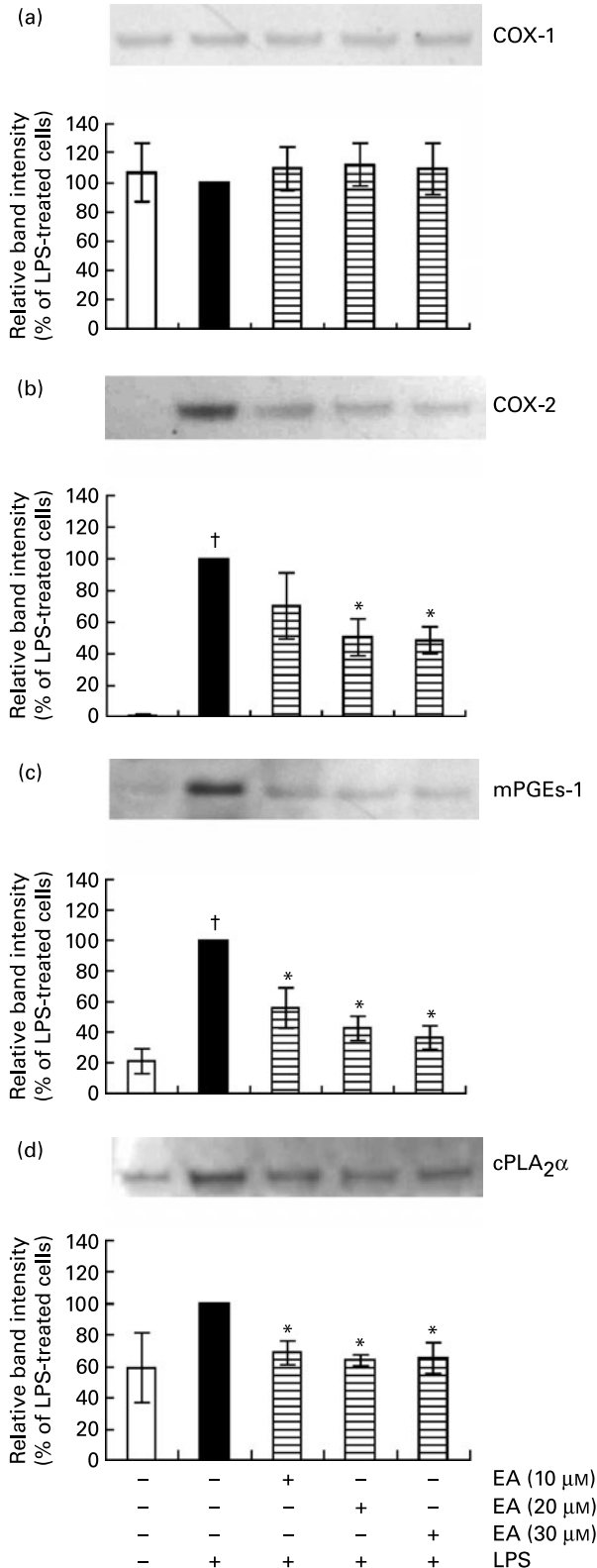
Fig. 2. Effect of ellagic acid (EA) on protein expression of cyclo-oxygenase (COX)-1, COX-2, microsomal PGE synthase-1 (mPGEs-1) and cytosolic phospholipase A$_2$α (cPLA$_2$α) in monocytes. Protein expression of COX-1 (a), COX-2 (b), mPGEs-1 (c) and cPLA$_2$α (d) in control cells and cells treated with lipopolysaccharide (LPS; 1 μg/ml) for 20 h in the prescence or abscence of 10, 20 or 30 μM-EA was analysed by immunoblotting. The relative band intensities were determined with Image Quant TL and intensity values of COX-2, mPGEs-1 and cPLA$_2$α were normalised to the corresponding value of COX-1 and are expressed as percentage of the response of LPS alone. Values are means from five individual experiments, with standard errors represented by vertical bars. * Significant inhibition v. LPS-treated cells ($P < 0.05$). † Significant induction v. control cells ($P < 0.05$).

shown to inhibit several different protein kinases. Among these, extracellular signal-regulated protein kinase[17,18], p38[18] and c-Jun N-terminal kinase[18] are of special interest since they have been suggested to participate in the regulation of the expression of both COX-2[34–36] and mPGEs-1[37]. Regarding transcription factors, ellagic acid has been shown to inhibit activation of NFκB in response to LPS stimulation of human intestinal CaCo2 cells[20], IL-1β stimulation of endothelial cells[16] and human pancreatic adenocarcinoma cells[19]. This is of interest since the promoter region of both cPLA$_2\alpha$ and COX-2 have binding sites for NFκB[38]. NFκB activation has been shown to mediate LPS-induced expression of COX-2 in macrophages[39] and also cPLA$_2\alpha$ expression has been suggested to be mediated via NFκB[40,41]. IL-1β-induced expression of mPGEs-1 in chondrocytes has also been suggested to be regulated via NFκB[42]. However, no binding

site for NFκB has been found in the promoter region of mPGEs-1 and it has therefore been suggested that the effect of NFκB is mediated via activation of the transcription factor early growth response-1[42], which has been shown to induce expression of mPGEs-1[43].

There are relatively few reports regarding the absorption and metabolism of ellagic acid in response to dietary intake of fruits and juices containing ellagic acid in human subjects. Seeram et al.[44] have reported submicromolar levels of ellagic acid to be present in plasma after consumption of 180 ml pomegranate juice (containing 12 mg free ellagic acid). This is a lower concentration of ellagic acid than the 5–30 μM used in the present study on monocytes and also lower than that used in several other cellular studies[16,18–20,45]. However, no studies on the uptake have been performed after intake of higher doses of pomegranate juice or pure ellagic acid, which potentially could result in higher levels in plasma. Of relevance for potential effects of ellagic acid on inflammatory reactions, consumption of pomegranate juice has been shown to reduce LDL oxidation[46], systemic blood pressure[47] and stress-induced ischaemia[48]. All of these inflammatory responses could potentially involve PGE$_2$.

In conclusion, we have shown that ellagic acid inhibits PGE$_2$ release in human monocytes and that this inhibition is mediated by an inhibition of the LPS-induced expression of cPLA$_2\alpha$, COX-2 and mPGEs-1 proteins and not by a direct effect on the activity of the enzymes. Adams et al.[45] have previously shown that pomegranate juice inhibited TNFα-induced expression of COX-2 in HT 29 colon cancer cells. Pomegranate juice contains ellagic acid but also other bioactive polyphenols, such as flavonoids, that may have contributed to the inhibition of COX-2 expression in the
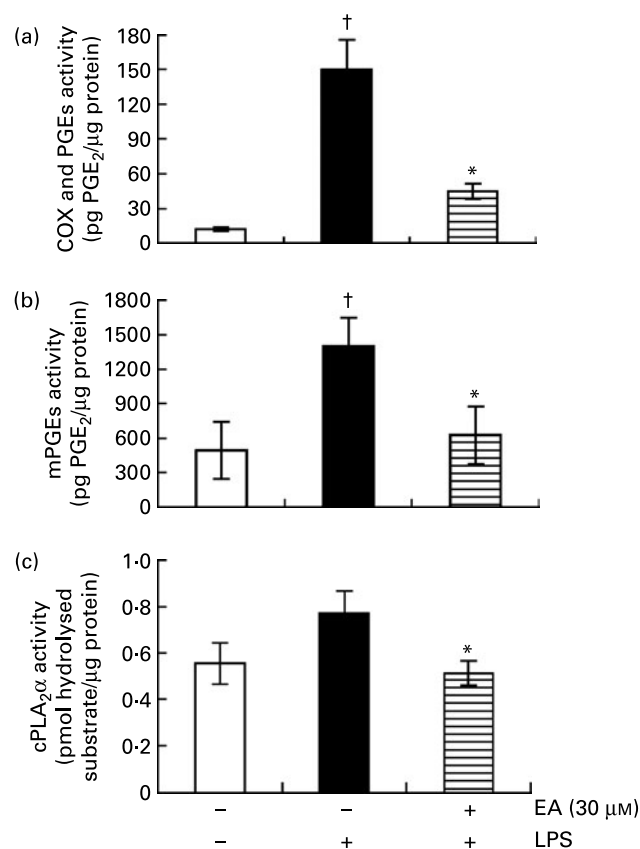
Fig. 3. Activity of cyclo-oxygenase (COX) and PGE synthase (PGEs), microsomal PGEs (mPGEs) and cytosolic phospholipase A$_{2\alpha}$ (cPLA$_{2\alpha}$) in subcellular fractions from control, lipopolysaccharide (LPS)- and LPS plus ellagic acid (EA)-treated monocytes. Cells were left untreated (control), treated with LPS (1 μg/ml) alone for 20 h or treated with 30 μM-EA for 21 h in combination with LPS. Subcellular fractions were prepared as described in Materials and methods and assayed for combined COX and PGEs activity in 1700 g supernatant fractions using arachidonic acid (AA) as the substrate (a), mPGEs activity in the microsomal fraction using PGH$_2$ as the substrate (b) and cPLA$_{2\alpha}$ activity in cytosolic fractions using sonicated vesicles of 1-stearoyl-2[14C]arachidonoyl-phosphatidylcholine ([14C]AAPC) as the substrate (c). Results are presented as pg PGE$_2$/μg protein for COX and PGEs activity and as pmol hydrolysed AA/μg protein for cPLA$_{2\alpha}$ activity. Values are means from three individual experiments in (a) and from four individual experiments in (b) and (c), with standard errors represented by vertical bars. * Significant inhibition v. LPS-treated cells ($P < 0.05$). † Significant induction v. control cells ($P < 0.05$).
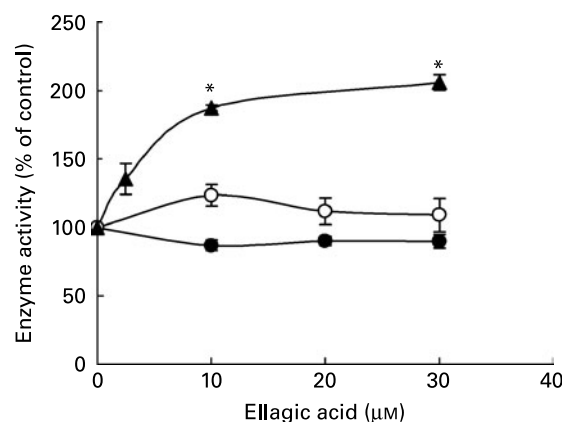
Fig. 4. Effects of ellagic acid on the enzymic activity of cyclo-oxygenase (COX) and PGE synthase (PGEs), microsomal PGEs (mPGEs) and cytosolic phospholipase A$_{2\alpha}$ (cPLA$_{2\alpha}$). Subcellular fractions from cells treated with lipopolysaccharide were analysed with or without the addition of ellagic acid (2·5–30 μM) in the reaction mixture. Subcellular fractions (prepared as described in Materials and methods) were assayed for combined COX and PGEs activity (▲) in 1700 g supernatant fractions using arachidonic acid as the substrate, mPGEs activity (○) in the microsomal fraction using PGH$_2$ as the substrate and cPLA$_{2\alpha}$ activity (●) in the cytosolic fraction using sonicated vesicles of 1-stearoyl-2[14C]arachidonoyl-phosphatidylcholine ([14C]AAPC) as the substrate. Results are presented as percentage of control (no addition of ellagic acid). Values are means from three (combined COX and PGEs activity) or four (mPGEs and cPLA$_{2\alpha}$ activity) individual experiments, with standard errors represented by vertical bars. * Significant increase in activity compared with control ($P < 0.05$).

colon cancer cells. However, the present study is to our knowledge the first report to show effects of ellagic acid on the expression of enzymes involved in the formation of PGE_2. Future studies are required to elucidate the mechanisms, such as effects on protein kinases and transcription factors, by which ellagic acid inhibits the LPS-induced expression of prostaglandin-synthesising enzymes.

Acknowledgements

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